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(57) Abstract

A polynucleotide molecule *dlk* is expressed in neuroendocrine tumors, including small cell lung carcinoma. A Dlk polypeptide encoded by *dlk* polynucleotide molecule can be used in detecting the existence of a primary or secondary neuroendocrine tumor. Monoclonal antibodies are produced against Dlk which are useful for detection and therapy of a neuroendocrine tumor.

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DELTA-LIKE GENE EXPRESSED IN NEUROENDOCRINE TUMORS

Background of the Invention

The expression of genes during the development of a pluripotent or progenitor cell into a differentiated, mature cell can provide a context for the study of tumorigenic cells whose origin is derived from such progenitor cells. In certain hematopoietic or epithelial tumors, malignant gene expression correlates substantially with the expression observed during normal development of the tissue from which the tumor originates, Gordon et al., *J. Cell Biol.* **108**: 1187 (1989); Godal et al., *Adv. Cancer Res.* **36**: 211 (1982). In fact, many biological activities of progenitor cells, including cellular migration and tissue remodeling, resemble pathological activities of cancer cells, such as metastases and tumor invasion.

Neuroblastoma, a tumor of the adrenal gland which afflicts persons during early childhood, is another system in which tumor biology correlates with that of normal differentiation and morphogenesis of its progenitor cells (neuroblast). Neuroblastoma is an embryonal tumor that exhibits both undifferentiated and differentiated histopathology. The development of neuroblastoma tumors mimics stages identifiable during histogenesis of its tissue of origin, the adrenal medulla. Cooper et al., *Cell Growth and Diff.* **1**: 149 (1989).

During the development of human adrenal medulla neuroblasts into mature chromaffin cells, four individual genes are expressed in a sequential pattern. Once a neuroblast is induced to differentiate along a neuroendocrine pathway, the progressive stages of chromaffin maturation are marked by a temporal expression of genes denoted TH, CGA, pG2 and B2M (Cooper, *supra.* at page 153). Cooper found that the pattern of gene expression of these four markers in neuroblastoma cells mimics that of normal adrenal neuroblast arrested during three different stages of development.

One of these marker genes, pG2, was identified first in pheochromocytoma, a tumor of the adult adrenal medulla (Helman

et al., *PNAS USA* **84**: 2336 (1987)). Helman reported that pG2 also is highly expressed normal human adrenal cells.

Helman isolated a full-length cDNA from a human adrenal cDNA library, and identified a corresponding pG2 protein containing 286 amino acids, having a predicted molecular weight of 30.6 kilodaltons (kDa) (Helman et al., *Nucleic Acids Res.* **18(3)**: 685 (1990)).

A gene having developmentally-regulated expression, paralleling that of pG2, would be useful for detecting pheochromocytoma or neuroblastoma by genetic methods, especially since pG2 expression is restricted to the adrenal gland in non-malignant tissue.

Summary of the Invention

It is therefore an object of the present invention to provide a newly-isolated polynucleotide molecule, *dlk*, which can be employed in genetic assays to provide a method for detection of a primary or secondary pheochromocytoma or neuroblastoma, or identification of a stage of these tumors.

It is also an object of the present invention to provide a method for detecting primary or secondary small cell lung carcinoma (hereafter, SCLC) or for staging tumor progression of SCLC, which employs *dlk* polynucleotide molecules in genetic assays.

It is a further object to provide a polynucleotide molecule, designated *dlk*, which encodes a corresponding Dlk polypeptide. Dlk polypeptides are useful for generating monoclonal or polyclonal antibodies having specificity for an epitope of the Dlk polypeptide.

Dlk-specific antibodies, and in particular, labeled monoclonal Dlk-specific antibodies, are useful for detection of primary or secondary neuroendocrine tumors. According to the present invention, Dlk-specific monoclonal antibodies conjugated to a toxin are useful for treatment of primary or secondary neuroendocrine tumors, as well.

In accomplishing these and other objects of the invention, there has been provided, in accordance with one

aspect of the present invention, an isolated polynucleotide molecule comprising a DNA sequence encoding a Dlk polypeptide.

An object of the present invention is to provide an isolated Dlk polypeptide consisting essentially of the amino acid sequence shown in Figure 1B, or in Figure 1A.

Another object of the present invention is to provide an isolated polynucleotide molecule which encodes a human or mouse Dlk polypeptide consisting essentially of the amino acid sequence shown in Figures 1B, or 1A, respectively.

A further object of the invention is to provide a method for detecting a tumor which expresses dlk, including the steps of contacting RNA from a sample suspected of being tumorigenic with a *dlk* polynucleotide molecule, under conditions permissive of hybridization between *dlk* polynucleotide molecule and the sample, and detecting hybridization between the polynucleotide molecule and sample.

Yet another object of the invention is to provide a method for detecting a small cell lung carcinoma, including the steps of contacting RNA from a sample of bronchial epithelial cells suspected of being tumorigenic with *dlk* polynucleotide molecules, under conditions permissive of hybridization between dlk polynucleotide molecules and the sample, and detecting hybridization between the polynucleotide molecules and sample.

Brief Description of the Drawings

Figure 1 shows an alignment of mouse (Figure 1A) and human (Figure 1B) Dlk amino acid sequences. Identical amino acids are shown by the character (|). Similar amino acids are indicated by (^) and classified into the following groups: A, S & T; D & E; N & Q; R & K; I, L, M & V; and F, Y & W. Potential biologically significant sites, found in the database PROSITE (accessible commercially through Intelligenetics Inc. (Mountain View, CA)), are indicated by numbers: 1. N-glycosylation site; 2. Protein Kinase C phosphorylation site; 3. N-myristylation site; 4. Aspartic acid and asparagine hydroxylation site. Potential sites of cleavage in the signal peptide are indicated by (*).

Figure 2 shows the human *dlk* DNA sequence.

Figure 3 shows the mouse *dlk* DNA sequence.

Figure 4 shows an alignment of a consensus sequence of *dlk* EGF-like repeats with EGF-repeats found in several invertebrate homeotic genes. As described in Example 4, a *dlk* EGF-like repeat consensus sequence was obtained by alignment of 12 EGF-like repeats of *dlk* from both human and mouse. This consensus sequence then was aligned with the consensus sequences of several invertebrate homeotic genes (similarly obtained) and mouse EGF.

Detailed Description of the Preferred Embodiments

A human polynucleotide molecule, *dlk*, and a corresponding human polypeptide, Dlk, encoded by *dlk*, were discovered, isolated and characterized. The human *dlk* polynucleotide molecule was found to be expressed in pheochromocytoma, neuroblastoma, and SCLC tumors.

Dlk protein is about 383 amino acids in length and has a molecular weight of about 42 kDa. In addition to human *dlk*, other polynucleotide molecules belonging to the *dlk* family are provided according to the invention, including murine *dlk* (Figure 3) and a human variant-*dlk*, isolated from placenta as described herein.

According to the present invention, isolated polynucleotide molecules or fragments thereof belonging to the *dlk* family are useful in the detection of SCLC and neuroendocrine cancers. The expression patterns of *dlk* can be exploited both (1) to detect primary or secondary tumor cells by the presence of *dlk* and (2) to diagnose the stage of a tumor that expresses *dlk*, by measuring the level of *dlk* expression.

Dlk is a transmembrane protein having an expression pattern, in normal non-fetal tissues, which is restricted to the adrenal gland. As a consequence, Dlk is a readily accessible target for antibody imaging or therapy of SCLC, pheochromocytoma and neuroblastoma tumors. According to the present invention, antibodies having specificity for the Dlk

protein are made and employed to detect or treat cells which produce the Dlk protein.

Human *dlk* cDNA comprises a polynucleotide molecule having the sequence shown in Figure 2, as determined by nucleotide sequence analysis. The open reading frame, nucleotides 174(ATG) - 1322(TAA), is 1149 nucleotides long. The mouse *dlk* polynucleotide molecule comprises a DNA sequence having an open reading frame, nucleotides 134(ATG) - 1288(TAA), of 1155 nucleotides, as shown in Figure 3. The murine Dlk protein is about 385 amino acids and has a molecular weight of about 42 kDa.

According to the present invention, a variant of human Dlk is identified in which an amino acid is deleted. A cDNA encoding "variant-Dlk," in which amino acid number 347 of the amino acid sequence shown in Figure 1(B) is deleted, was isolated from a total human placental cDNA library. Amino acid position 347 occurs in an intracellular domain of the protein. The placental library containing variant-dlk also contained substantial amounts of the non-variant form, that is, *dlk* polynucleotide molecule shown in Figure 1B.

The *dlk* polynucleotide molecule was identified by examination of cDNA expression products of human SCLC (hSCLC) lines which were responsive to stimulation with the ligand, gastrin-releasing peptide (GRP), a neuropeptide implicated in the release of gastrin through its interaction with a G-protein-coupled receptor, GRP receptor. GRP (peptide) is a mitogen for both normal lung epithelial cells and SCLC cells, and for murine Swiss 3T3 fibroblasts.

GRP-responsive hSCLC lines were compared with murine fibroblast cell lines that were differentially responsive to GRP. This approach, as detailed in Example 1, yielded a partial length cDNA molecule that hybridized a 1.6 Kb mRNA expressed both in responsive fibroblasts and responsive SCLC lines. A commercially available Swiss 3T3 fibroblast cDNA library was screened with the partial length cDNA, which yielded several clones having 1.6 Kb inserts, which then were sequenced.

A computer search of the databases "Swissprot" and "NBRF Protein," described by Devereux et al., *Nuc. Acids Res.* **12(1)**: 387 (1984), indicated a high degree of homology between Dlk and proteins encoded by several homeotic genes, identified in Example 3. Homeotic genes are development-controlling regulatory genes that assign spatial identity to groups of cells with respect to their morphogenic fates. In segmented organisms, for example, homeotic genes are required for the proper morphogenesis of a distinct region (such as a leg, or antennae) and act by controlling the activities of other genes during development. The Dlk protein of the present invention exhibited highest homology with the protein Delta, a neurogenic locus involved in normal neural differentiation in *Drosophila*. Thus, the present protein was designated "Dlk" for being "delta-like."

Mouse and human Dlk protein sequences share 86.2% identity as well as many potential sites of biological importance, including 6 epidermal growth factor (EGF)-like repeats, a transmembrane domain, and a signal peptide domain at the amino terminus. Based upon these structural features, *dlk* appears to be a new member of the family of EGF-like neurogenic genes of *Drosophila*, which are involved in developmental decisions of the embryonal ectoderm to differentiate into epidermal or neuronal cells.

The expression pattern of *dlk* and its sequence homology with homeotic proteins support the notion that *dlk* functions in the differentiation pathway for cells of the chromaffin lineage. As detailed in Example 2, *dlk* is expressed in primary and secondary pheochromocytoma and neuroblastoma cells, and in normal (nonhistopathological) human adrenal medulla and placental cells. According to the present invention, SCLC and neuroblastoma are the only tumors known to express *dlk* as a function of differentiation.

An isolated *dlk*, *dlk*-variant, and murine *dlk* polynucleotide and protein products are employed in diagnostic methods (described further below) and are made according to the following description. Hereafter, the techniques and

applications described for *dlk* polynucleotide molecule (DNA, RNA) and Dlk protein are intended to be useful for DNA, RNA and protein of murine *dlk*, and of variant-*dlk*, as well.

A Dlk polypeptide, according to the present invention, is produced by recombinant DNA techniques, such as those described by Maniatis et al., MOLECULAR CLONING - A LABORATORY MANUAL, Cold Spring Harbor Laboratory (1982). Methods specifically applicable to cloning the *dlk* polynucleotide molecule are described in Example 1.

The *dlk* polynucleotide molecule of Figure 1B can be cloned into suitable expression vectors and expressed in prokaryotic, insect or eukaryotic expression systems, including *Baculovirus* or *E. coli* (Boehringer Manhein). Using conventional techniques, therefore, a polynucleotide sequence encoding a Dlk protein, can be obtained as a cDNA from mRNA from a commercial adrenal medulla or Swiss 3T3 fibroblast library, or from SCLC, neuroblastoma or pheochromocytoma cell lines. The mRNA can be converted to double-stranded DNA using cDNA cloning techniques well-known to the art, including PCR-based techniques. Linkers or tails may be added to the ends of the double-stranded DNA to provide convenient restriction sites. After restriction enzyme digestion, the DNA may be introduced into a cloning vector, such as a plasmid, which has been digested with a restriction enzyme that generates appropriately compatible ends. A suitable plasmid vector in this context is pGEX-λ (Pharmacia). Following ligation, by means of standard techniques, the DNA is introduced into a cell, where its expression produces the desired protein.

Alternatively, a Dlk polypeptide is produced using a commercially available *in vitro* translation kit from NEN (Boston, MA), as detailed in Example 1. This kit employs a translation system (including ribosomes, polymerases, amino acids, etc.) derived from rabbit reticulocyte lysates.

The term "isolated," with reference to the *dlk* polynucleotide molecule indicates that such a molecule is free of the proteins with which it is normally associated, such as histones. An isolated form of the *dlk* is substantially free

of other DNA that does not function to regulate, promote, enhance or otherwise modulate its expression.

The term "isolated," with reference to Dlk protein, connotes a polypeptide that is free of other proteins with which it is normally associated.

An isolated *dlk* polynucleotide molecule is useful for detecting primary SCLC and the metastatic spread of SCLC and other neuroendocrine cancers. More specifically, the present invention provides a method of tumor detection which includes the steps of contacting a sample suspected of containing a tumor with a *dlk* polynucleotide molecule, and detecting expression of *dlk* polynucleotide products (DNA, RNA, mRNA) in non-adrenal cells. Detection of a *dlk* polynucleotide product identifies the cells as metastatic cells (secondary tumor) of neuroblastoma, pheochromocytoma or SCLC, or as a primary tumor of SCLC.

The ability to detect *dlk*-expressing cells is useful for both tumor detection and tumor identification. After detecting *dlk*-expression, a tumor type is determined by detecting either a tumor-specific marker, tumor-specific morphology, or by presentation by the patient of a clinical pathology that is distinctly associated with any of the tumors selected from the group including neuroblastoma, pheochromocytoma or SCLC. For example, information such as the identification of a cellular marker, histological feature or disease symptom which is specific to one of the tumors of neuroblastoma, pheochromocytoma or SCLC, is recognized.

If *dlk* expression is detected in cells of a sample taken from bronchial epithelial tissue or tissue removed from the lung, the observation identifies a primary SCLC. It is preferred that a second step of confirming the origin of the detected *dlk*-expressing tumor cells as SCLC be performed by detection of a marker, histological feature, or presentation of a distinctive symptom associated with this tumor. For example the histology of an "oat cell" commonly associated with SCLC is detected to confirm the presence of SCLC.

The expression of *dlk* is detected by hybridization with a *dlk* polynucleotide probe. This method includes the steps of contacting a suspected tumor sample with a *dlk* polynucleotide molecule, and detecting the hybridization between the polynucleotide molecule and sample. A positive hybridization signal indicates that the sample is of tumor origin.

The polynucleotide molecule or "*dlk* probe" used to detect *dlk* expression is a labeled fragment of *dlk*, or preferably a full-length *dlk* DNA molecule which will hybridize to mRNA or DNA from normal adrenal and neuroendocrine tumor cells. Probes complementary to *dlk* are prepared by conventional methods, and are preferably allowed to hybridize to mRNA or DNA, using conventional *in situ* hybridization techniques. Unhybridized probe is removed by nuclease digestion.

In situ hybridization techniques which are known in the art may employ the use of fluorescent labels and radiolabels which can be easily quantitated by fluorescence microscopy or autoradiography, respectively. Generally, fluorescent labels will be preferred. Another labelling technique may employ enzymatic tags which generate readily quantifiable colorimetric or chemiluminescent signals. The hybridization intensity detected in their procedures reflects the amount of *dlk* within the biological sample.

The *dlk* polynucleotide of the invention can be employed as a probe in RNA ("Northern") blotting procedures. According to this method, RNA is first isolated from tissue by any of a number of standard procedures (Lehrach, H., *Biochemistry*, **16**: 4743 (1975)). The RNA sample is then subjected to denaturing gel electrophoresis and is transferred to a nitrocellulose membrane or other solid support matrix. The *dlk* mRNA can be detected by hybridization of radioactively or non-radioactively labelled *dlk*, or *dlk* fragments, preferably under high stringency conditions as would be familiar to one of ordinary skill in the art. The amount of hybridization can be quantified by densitometry.

In yet another embodiment of the present invention, the polymerase chain reaction ("PCR") is used to detect *dlk* DNA or

mRNA in a sample. To perform PCR, a pair of *dlk* sequence specific polynucleotide primers is employed, which hybridize to opposite strands of the *dlk* gene at offset positions on the double helix. Such primers, taken from the *dlk* polynucleotide sequences provided in accordance with the invention, represent fragments which preferably are unique to *dlk*, e.g., sequences having low homology with other proteins than Dlk. Two exemplary *dlk*-specific primer sequences useful in this context include the following sequences, which encode a portion of the intracellular region of Dlk:

5'-CAA GCC CGA GTT CAC AGG TC-3'

5'-TCG GGG AAG ATG TTG AC-3'.

Other such primer pairs can be selected and utilized, as well.

The primers provide initiation points for DNA synthesis. In the presence of DNA polymerase, the four deoxynucleotide triphosphates ("dNTPs") and other necessary cofactors, all of which are well known to the art, new DNA strands are synthesized complementary to the templates which hybridized with the primers. Several rounds of synthesis are carried out, with allowance for denaturation of the double stranded products between rounds. Preferably, a thermal stable DNA polymerase is used so that it is not necessary to add enzyme anew for each round of synthesis.

The PCR produces a double stranded DNA amplification product which has the same sequence as the original stretch of the *dlk* DNA defined by the ends of the primer pair sequences. The amount of PCR product indicates the amount of *dlk* DNA or *dlk* mRNA in the sample. The product can be detected by a variety of methods well-known in the art. The PCR products can be resolved by agarose or polyacrylamide electrophoresis and detected by fluorescence staining, such as ethidium bromide. Alternatively, one of the dNTPs may be labelled and the PCR products may be determined by measuring incorporation of the labelled dNTP. A variety of other methods for resolving, detecting and quantitating PCR products are well-known to those of ordinary skill in the art.

The PCR may be made specific for either *dlk* DNA or *dlk* mRNA. For instance, RNAse or DNAse may be used to remove one template or the other from the sample, and the use of primers that distinguish between the gene and the message, for example, a primer that hybridizes to a sequence in the untranscribed region of the promoter will be specific for the *dlk* gene, and not for the *dlk* mRNA.

Other techniques suitable to the claimed methods are readily apparent to the skilled artisan and can include Nuclease Protection Assays, ELISA and Western blotting. Several assay techniques which are based upon immunological reactions between antigens and antibodies are contemplated by the invention. In particular, assays which use antibodies having specificity for Dlk protein are useful to detect cells which produce Dlk protein.

Antibodies having specificity for Dlk-expressing cells are obtained by immunizing an animal with Dlk protein. In this context, the term "antibody" encompasses both monoclonal and polyclonal antibodies. such an antibody can belong to any antibody class (IgG, IgM, IgA, etc.). According to the present invention, an entire Dlk polypeptide is injected into an animal for the purpose of obtaining polyclonal antibodies, or for obtaining lymphocytes or spleen cells for production of monoclonal antibodies.

The general techniques of monoclonal antibody (MAb) production, such as those described by Kohler and Milstein, *Nature* **256**:495 (1975), are applied to produce a monoclonal antibody having specificity for Dlk protein. This procedure includes the steps of isolating lymphocytes of an animal which has been sensitized or injected with Dlk polypeptide, fusing them with a myeloma partner to produce hybridomas, then screening the hybridomas for production of "anti-Dlk antibodies" which exhibit binding specificity for a Dlk polypeptide.

The term "Antibody" also encompasses fragments, like FAb and $F(Ab^1)_2$, of anti-Dlk antibodies, and conjugates of such fragments, and so-called "antigen binding proteins" (single-

chain antibodies) which are based on anti-Dlk antibodies, in accordance, for example, with U.S. patent No. 4,704,692, the contents of which are hereby incorporated by reference. Alternatively, MAbs or fragments thereof can be produced via the expression of genes which encode variable regions of such an MAb in host cells like *E. coli*, see, e.g., Ward et al., *Nature* **341**: 544-546 (1989), or transfected murine myeloma cells. See Verhoyen et al., *BioAssays* **8**: 74 (1988); Gillies et al., *Biotechnol.* **7**: 799-804 (1989); Nakatani et al., *Biotechnol.* **7**: 805-10 (1989).

Assays in which the above antibodies are employed can include enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays, immunoelectrophoresis, and the like. Also useful diagnostically are immunohistochemical techniques which employ monoclonal antibodies of known reactivity.

In accordance with this aspect of the present invention, a sample is obtained from a person to detect: (1) a small cell lung carcinoma, by removing a body fluid or tissue suspected of harboring a tumor, such as alveolar, bronchiolar, or respiratory epithelial cells obtained from a bronchial wash, nasopharyngeal aspirates, throat swabs or the like; (2) a metastasized neuroendocrine tumor, by biopsy, taken from tissue other than the adrenal gland (including cortex and medulla. Immuno-histochemical studies can be performed on such cells using a monoclonal antibody specific for Dlk.

Diagnostic applications of these antibodies are exemplified, according to the present invention, by the use of a kit containing an anti-Dlk antibody, which undergoes a reaction with a biological sample to detect Dlk protein. Such a reaction involves the binding of anti-Dlk antibody to Dlk antigen, under conditions permissive of binding. The observation of an antibody-antigen complex in a biological sample indicates a positive result. A kit of this sort could be used to detect the extent of expression of Dlk in a particular biological sample from an individual, animal, or cell line.

Such an immunodiagnostic kit can include anti-Dlk antibody and a receptacle for containing the antibody in a sterilized form. The kit can further include anti-isotype serum antibody which recognizes the anti-Dlk antibody (Fc portion) and which is conjugated to a label, such as an enzyme or fluorescent moiety.

In a preferred embodiment, a radiolabeled anti-Dlk antibody is provided. Such an antibody, preferably a monoclonal antibody, is administered to an animal or person for imaging purposes. After a suitable period of time for the administered antibodies to bind Dlk expressing cells, a gamma camera machine is applied to detect the presence of labeled antibodies within the organism. Such a procedure provides information as to where in the organism a primary or secondary Dlk-expressing neuroendocrine tumor is located.

A therapeutic application of anti-Dlk monoclonal antibodies includes administration of anti-Dlk immunotoxins. Conjugation of an anti-Dlk monoclonal antibody to a toxin, such as *Pseudomonas* exotoxin or other toxins commonly conjugated to an antibody by means of a conventional antibody-toxin linkage. Hertler et al., *J. Clin. Oncol.* **7(12)**: 1932 (1989), describe methodologies for creating an antibody-toxin linkage, and is incorporated by reference herein. Thus, the anti-Dlk monoclonal antibody-toxin conjugates described are administered to an individual to target and selectively kill Dlk-expressing cells present in neuroendocrine tumors.

Similarly, a kit is provided which contains anti-Dlk immunotoxins in a receptacle. A kit can include the anti-Dlk immunotoxins and a pharmaceutical excipient in a receptacle.

The present invention is further described with reference to the following, illustrative examples. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of the invention. Although any method and materials similar or equivalent to those described herein can be used in the practice of the invention, the preferred methods and materials have been described. Unless mentioned

otherwise, the techniques employed or contemplated herein are standard methodologies known to the art. The materials, methods and examples are illustrative only and not limiting.

Example 1. IDENTIFICATION OF *dlk* POLYNUCLEOTIDE AND POLYPEPTIDE MOLECULES

Identification of *dlk*

In investigating molecules associated with the gastrin-releasing peptide (GRP) responsive phenotype, genes were identified which both were: (1) expressed differentially between responsive murine Swiss and unresponsive murine Balb/c 3T3 fibroblasts, and (2) expressed in GRP-responsive human SCLC cell lines. The rationale for this approach was that gene products which correlated with a GRP-responsive phenotype would be missing from Balb/c and unresponsive SCLC cell lines, but present in Swiss 3T3 fibroblasts and responsive SCLC cell lines.

A differential cDNA library was constructed that was enriched for clones expressed in Swiss 3T3 but not in Balb/c 3T3 fibroblasts. The differential library of Swiss 3T3 compared with Balb/c 3T3 fibroblasts was constructed as explained in detail in Timblin et al., *Nucleic Acids Res.* **18**: 1587 (1990). The RNA isolation, electrophoresis, northern blots, and hybridization techniques were performed as described in Davis, et al., BASIC METHODS IN MOLECULAR BIOLOGY, Elsevier, New York, (1986). The nucleic acid probes were labelled with ^{32}P dCTP (Amersham, Arlington Heights, IL) by random priming as described in Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley and Sons, New York 3.5.9.-3.5.10 (1991).

A partial length clone (150 nucleotides long) isolated from this differential library hybridized with a 1.6 kilobase mRNA which exhibited an expression pattern consistent with the two screening requirements. This partial length clone was then used to screen a commercially available oligo dT-primed cDNA library of Swiss 3T3 fibroblasts in the λZAPII vector (Stratagene (La Jolla, CA)), to obtain a full length clone.

Screening procedures and plasmid rescue of positive λZAPII clones were performed following the manufacturer's (Stratagene) protocol, as described by Short et al., *Nuc. Acids Res.* **16**: 7583 (1988). Several clones with inserts of approximately 1.6 kilobase pairs in length were obtained from this screening procedure.

DNA Sequencing

Rescued plasmids were sequenced with Sequenase (USB, Cleveland, OH) by the dideoxy chain termination method, according to the manufacturer's protocol described by Tabor et al., *J. Biol. Chem.* **214**: 6447 (1989). Nucleotide sequence analysis defined an open reading frame of 1155 nucleotides, encoding a putative protein (Dlk) of 385 amino acids with a molecular weight of 41,320 daltons. This open reading frame was classified as coding by both Fickett's and Shepherd's methods. Fickett et al, *Nucleic Acids Res.* **10**: 5303 (1982); Shepherd et al., *Meth. Enzymol.* **188**: 180 (1990). The open reading frames were identified by software programs implementing these methods (PC/Gene software package, Intelligenetics Inc. (Mountain View, CA); A. Bairoch, Ph.D thesis, University of Geneva, (1990)).

In vitro Translation of Dlk Polypeptide

In vitro translation assays from mouse *dlk* mRNA were performed using a rabbit reticulocyte lysate system from NEN (Boston, MA), according to manufacturer's protocol, as described by Lockhard et al., *Biochem. Biophys. Res. Comm.* **37**: 204 (1969).

The *dlk* mRNA was selected by hybridization of poly A+ RNA from Swiss 3T3 fibroblasts with denatured full-length *dlk* immobilized on nitrocellulose filters. (*dlk* mRNA was selected by hybridization of 2 μg of poly A+ Swiss 3T3 RNA with 5 μg of nitrocellulose-immobilized denatured *dlk*.) The bound RNA was eluted by boiling. Mouse *dlk* mRNA was also prepared *in vitro* using two different full length *dlk* cDNAs cloned in pGEM4Z (Promega). These three mRNAs were used as templates for *in vitro* translation.

Labelled proteins were analyzed in a 12% polyacrylamide gel followed by fluorography. A protein band of approximately 42 kilodaltons was present in all three samples, in agreement with the predicted molecular weight of *Dlk* polypeptide.

A Comparison between mice and humans

Mouse and human *dlk* polynucleotide sequences are 86.2% identical and 90.1% similarity in their amino acid sequence. They share many potential sites of biological activity, including 6 EGF-like repeats (highly homologous to those found in invertebrate neurogenic proteins) an integral transmembrane domain and a signal peptide domain.

The structural characteristics of *dlk* were analyzed with the program PC/Gene (Intelligenetics Inc. (Mountain View, CA), A. Bairoch, Ph.D thesis, University of Geneva (1990)). The transmembrane domain was identified using the program RAOARGOS, implementing the method of Rao and Argos, *Biochim. Biophy. Acta* **869**: 197 (1986). The signal peptide was analyzed with the program PSIGNAL, according to the method of Von Heijne, *Nucleic Acids Res.* **14**: 4683 (1986).

Example 2. A COMPARISON BETWEEN pPG2 and *dlk* GENE EXPRESSION IN MICE, & *dlk* GENE EXPRESSION IN HUMANS

In normal tissues of human, mouse and hamster origin, *dlk* expression was detected according to the present invention, only in adrenal and placental tissue. Similarly, pG2 expression was known to be restricted to adrenal glands in normal human tissues.

The *dlk* mRNA was detected by Northern analysis in human and rat pheochromocytoma (PC12) cell lines. pG2 was identified in pheochromocytoma cell lines by Helman et al., *PNAS USA* **84**: 2336 (1987).

According to the present invention, *dlk* was detected in neuroblastoma (SK-N-SH) cells. pG2 expression in neuroblastoma cell lines was detected in differentiated cells, but absent from undifferentiated neuroblastoma cell lines. Cooper et al., *Cell Growth and Diff.* **1**: 149 (1989).

In addition, other cells which express *dlk* identified by the present invention include certain SCLC cell lines. Also, murine Swiss 3T3 fibroblasts were found to express *dlk* using a human *dlk* probe under high stringency conditions. Balb/c 3T3 fibroblasts were found to express *dlk* using a human *dlk* probe under high stringency conditions. Balb/c 3T3 fibroblasts RNA were negative for *dlk* expression under these conditions.

To explore the relationship between mouse *dlk* and human pG2, cDNA clones were isolated from a λgt10 human adrenal gland library (Clontech, Palo Alto, CA) using mouse *dlk* as a hybridization probe. Even under low stringency conditions, no clones were isolated which encoded proteins having structural characteristics similar to those reported for pG2. The cDNA inserts from positive λ clones were subcloned into pGEM4Z (Promega, Madison WI) and sequenced according to the method of Example 1. Polynucleotide sequence data from several full-length clones indicated that these cDNAs showed a 82.1% sequence identity with mouse *dlk* and encoded the human homolog of the mouse *dlk* protein (Fig. 1).

Dlk's structural characterization is very different from that predicted for pG2 protein (Helman et al., *supra*. (1987). the former protein consists of a 286 amino acid sequence (about 30 kDa), contains *no* EGF-like repeats and no signal peptide or transmembrane domains. This was so, despite a finding of an 81.2% nucleotide sequence identity of *dlk* with *pG2* is identified correctly as the *dlk* polynucleotide molecule shown in Figure 1.

Example 3. *dlk*/Dlk HOMOLOGY WITH OTHER GENES & PROTEINS

The *dlk* nucleic acid sequence shows a high degree of homology with the EGF-like neurogenic genes of *Drosophila*, which are involved in the decisions taken by the cells of the embryonal ectoderm to differentiate into epidermal or neuronal cells. Genes which were found to have highest homology to Dlk include: Delta, Notch and Serrate of *D. melanogaster*, lin-12 and glp1 of *C. elegans*, and uEGF1 of the sea urchin. Although

the degree of homology varied between the individual proteins and Dlk, regions of maximum homology exhibited up to 33% amino acid identity, which rose to around 75%, with allowance for conservative amino acid substitutions.

Figure 4 shows the alignment of mouse or human *dlk* EGF-like repetitive sequences with consensus sequences of EGF sequence repeats of several proteins. The alignment of the EGF-like repeats was done using the program CLUSTAL, described by Higgins et al., *Gene* **73**: 237 (1988). The sites of potential biological importance were analyzed with the program PROSITE. Residues highly conserved among homeotic genes also are conserved in *dlk*. This finding confirms that *dlk* is a member of the family of EGF-like homeotic genes. The amino acid sequence and structure of the EGF-like repeats, as well as the overall structure of *dlk*, are more related to the invertebrate homeotic genes than to other vertebrate non-homeotic EGF-like proteins, such as EGF-precursor, TGFα, the α, β1 and β2 chains of laminin, coagulation factors, or complement proteins, previously thought to be the mammalian counterparts of the invertebrate homeotic genes.

The *dlk* gene was detected in species ranging from birds to human, including: yeast, *Drosophilia*, *Xenopus*, mouse, rat, rabbit, chicken, dog, cow, monkey and human. However, despite the structural homology with invertebrate proteins, the *dlk* gene is absent from invertebrates and lower vertebrates.

The program PCOMPARE, described by Needleman et al., *Mol. Biol.* **48**: 443 (1970), included in PC/Gene was used for the analysis of homology. In this method, the optimal alignment score between two proteins were compared with the statistical distribution of 100 random alignments. An alignment score of greater than 5 positive standard deviations from the mean random alignment distribution was considered significant, particularly when no functional or structural relationship between the proteins compared is known. Representative alignment scores were determined: *Delta*, 20.2; *Serrate*, 19.7; *TAN-1*, 16.2; *Notch*, 14.6; *Xotch*, 13.6; *Drosophila* Laminin β2,

6.3; mouse Laminin β2, 4.1; human coagulation factor XII, 2.8; and human EGF precursor, 0.6.

Example 4. *dlk* EXPRESSION: NORTHERN BLOT mRNA ANALYSIS

Expression of *dlk* was detected by Northern analysis in SCLC lines NCI-H510, NCI-H69 and NCI-N592; in human neuroblastoma line SK-N-SH, and in the rat pheochromocytoma PC-12 cell line. Twenty μg of total RNA or 2 μg of poly A+ were separated in a 1% agarose gel and then blotted to a nitrocellulose filter (described in Example 1).

A 1.6 Kb band corresponding to *dlk* was observed only in the RNA samples from the SCLC cell lines NCI-N592, NCI-H69 and NCI-H510, and in Swiss 3T3 fibroblasts. Mouse Swiss 3T3 fibroblast RNA also showed a high degree of expression of *dlk*, even when the hybridization was performed under high stringency conditions using human *dlk* as a probe. Similar results were obtained using mouse *dlk* as a probe. Balb/c 3T3 fibroblast RNA was negative for *dlk* expression under these conditions. Ewing's sarcoma cell lines SK-ES-1, A4573 and TC106 did not express *dlk*.

In normal tissues of mouse, hamster, and human origin *dlk* expression was detected exclusively in the adrenal gland.

What Is Claimed Is:

1. An isolated Dlk polypeptide consisting essentially of the amino acid sequence shown in Figure 1B.

2. An isolated polynucleotide molecule which encodes a human Dlk polypeptide according to Claim 1.

3. An isolated polynucleotide molecule consisting essentially of the polynucleotide sequence shown in Figure 2.

4. An isolated polynucleotide molecule which encodes a murine Dlk polypeptide, wherein said Dlk polypeptide comprises the amino acid sequence shown in Figure 1A.

5. A method for detecting a tumor which expresses *dlk*, comprising the steps of

(a) contacting a sample suspected of being tumorigenic with *dlk* polynucleotide molecule, under conditions permissive of hybridization between *dlk* polynucleotide molecule and said sample and

(b) detecting the presence of hybridization between said polynucleotide molecule and said sample.

6. A method according to Claim 5, wherein said *dlk* polynucleotide molecule is an isolated polynucleotide molecule according to Claim 3.

7. A method according to Claim 5 for detecting a tumor identified as small cell lung carcinoma, wherein, in step (a), said sample comprises bronchial epithelial cells.

Figure 1A

Figure 1B

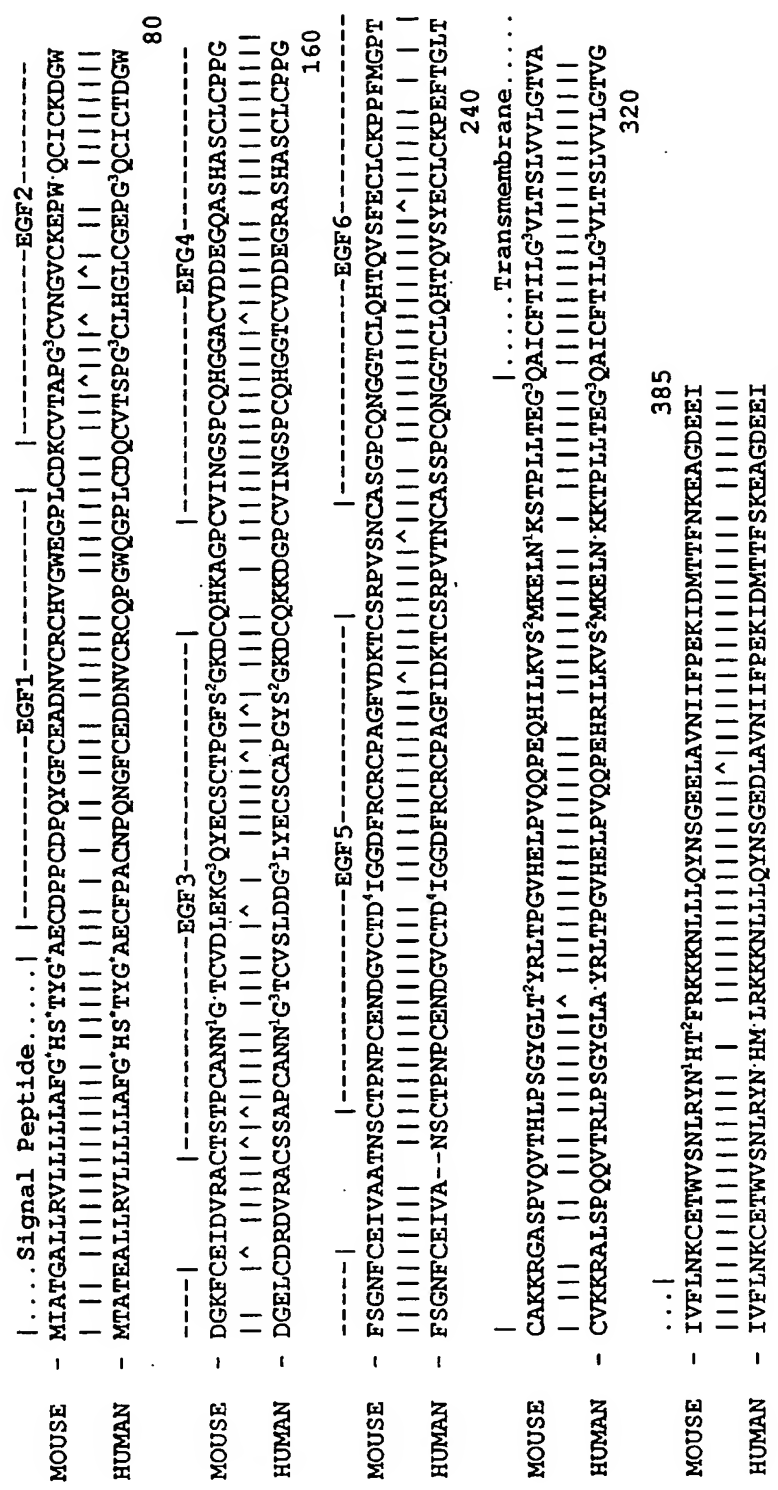

Figure 2

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        3       9       15      21      27      33      39      45
        |       |       |       |       |       |       |       |
    1 TCT AAA GGA GGT GGA GAG CGC ACC GCA GCC CGG TGC AGC CCG GTG
   46 CAG CCC TGG CTT TCC CCT CGC TGC GGC CCG TGC CCC CTT TCG CGT
   91 CCG CAA CCA GAA GCC CAG TGC GGC GCC AGG AGC CGG ACC CGC GCC
  136 CGC ACC GCT CCC GGG ACC GCG ACC CCG GCC GCC CAG AGA TGA CCG
  181 CGA CCG AAG CCC TCC TGC GCG TCC TCT TGC TCC TGC TGG CTT TCG
  226 GCC ACA GCA CCT ATG GGG CTG AAT GCT TCC CGG CCT GCA ACC CCC
  271 AAA ATG GAT TCT GCG AGG ATG ACA ATG TTT GCA GGT GCC AGC CTG
  316 GCT GGC AGG GTC CCC TTT GTG ACC AGT GCG TGA CCT CTC CCG GCT
  361 GCC TTC ACG GAC TCT GTG GAG AAC CCG GGC AGT GCA TTT GCA CCG
  406 ACG GCT GGG ACG GGG AGC TCT GTG ATA GAG ATG TTC GGG CCT GCT
  451 CCT CGG CCC CCT GTG CCA ACA ACG GGA CCT GCG TGA GCC TGG ACG
  496 ATG GCC TCT ATG AAT GCT CCT GTG CCC CCG GGT ACT CGG GAA AGG
  541 ACT GCC AGA AAA AGG ACG GGC CCT GTG TGA TCA ACG GCT CCC CCT
  586 GCC AGC ACG GAG GCA CCT GCG TGG ATG ATG AGG GCC GGG CCT CCC
  631 ATG CCT CCT GCC TGT GCC CCC CTG GCT TCT CAG GCA ATT TCT GCG
  676 AGA TCG TGG CCA ACA GCT GCA CCC CCA ACC CAT GCG AGA ACG ACG
  721 GCG TCT GCA CTG ACA TTG GGG GCG ACT TCC GCT GCC GGT GCC CAG
  766 CCG GCT TCA TCG ACA AGA CCT GCA GCC GCC CGG TGA CCA ACT GCG
  811 CCA GCA GCC CGT GCC AGA ACG GGG GCA CCT GCC TGC AGC ACA CCC
  856 AGG TGA GCT ACG AGT GTC TGT GCA AGC CCG AGT TCA CAG GTC TCA
  901 CCT GTG TCA AGA AGC GCG CGC TGA GCC CCC AGC AGG TCA CCC GTC
  946 TGC CCA GCG GCT ATG GGC TGG CCT ACC GCC TGA CCC CTG GGG TGC
  991 ACG AGC TGC CGG TGC AGC AGC CGG AGC ACC GCA TCC TGA AGG TGT
 1036 CCA TGA AAG AGC TCA ACA AGA AAA CCC CTC TCC TCA CCG AGG GCC
 1081 AGG CCA TCT GCT TCA CCA TCC TGG GCG TGC TCA CCA GCC TGG TGG
 1126 TGC TGG GCA CTG TGG GTA TCG TCT TCC TCA ACA AGT GCG AGA CCT
 1171 GGG TGT CCA ACC TGC GCT ACA ACC ACA TGC TGC GGA AGA AGA AGA
 1216 ACC TGC TGC TTC AGT ACA ACA GCG GGG AGG ACC TGG CCG TCA ACA
 1261 TCA TCT TCC CCG AGA AGA TCG ACA TGA CCA CCT TCA GCA AGG AGG
 1306 CCG GCG ACG AGG AGA TCT AAG CAG CGT TCC CAC AGC CCC CTC TAG
 1351 ATT CTT GGA GTT CCG CAG AGC TTA CTA TAC GCG GTC TGT CCT AAT
 1396 CTT TGT GGT GTT CGC TAT CTC TTG TGT CAA ATC TGG TGA ACG CTA
 1441 CGC TTA CAT ATA TTG TCT TTG TGC TGC TGT GTG ACA AAC GCA ATG
 1486 CAA AAA CAA TCC TCT TTC TCT CTC TTA ATG CAT GAT ACA GAA TAA
 1531 TAA TAA GAA TTT CAT CTT TAA ATG AG
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Total number of bases is: 1556.

Figure 3

```
        3       9       15      21      27      33      39      45
        |       |       |       |       |       |       |       |
    1 GGT GCA ACC CTA GCT TTC TTC CCG CTG GAC GCC CGT GCC CCC TTC
   46 GTG GTC CGC AAC CAG AAG CCC AGC GCA CGC CCC GGA GCA GCC CCT
   91 GCA CCG CCT CCG CTC CCC GGA CCG CGA CCC AGG CCG CCC CGA GAT
  136 GAT CGC GAC CGG AGC CCT CCT GCG CGT CCT CTT GCT CCT GCT GGC
  181 TTT CGG CCA CAG CAC CTA TGG GGC TGA ATG CGA CCC ACC CTG TGA
  226 CCC CCA GTA TGG ATT CTG CGA GGC TGA CAA TGT CTG CAG GTG CCA
  271 TGT TGG CTG GGA GGG TCC CCT CTG TGA CAA GTG TGT AAC TGC CCC
  316 TGG CTG TGT CAA TGG AGT CTG CAA GGA ACC ATG GCA GTG CAT CTG
  361 CAA GGA TGG CTG GGA CGG GAA ATT CTG CGA AAT AGA CGT TCG GGC
  406 TTG CAC CTC AAC CCC CTG CGC CAA CAA TGG AAC TTG CGT GGA CCT
  451 GGA GAA AGG CCA GTA CGA ATG CTC CTG CAC ACC TGG GTT CTC TGG
  496 AAA GGA CTG CCA GCA CAA GGC TGG GCC CTG CGT GAT CAA TGG TTC
  541 TCC CTG CCA GCA CGG AGG CGC CTG CGT GGA TGA TGA GGG CCA GGC
  586 CTC GCA TGC TTC CTG CCT GTG CCC CCC TGG CTT CTC AGG CAA CTT
  631 CTG TGA GAT CGT AGC CGC AAC CAA CAG CTG TAC CCC TAA CCC ATG
  676 CGA GAA CGA TGG CGT CTG CAC CGA CAT CGG GGG TGA CTT CCG TTG
  721 CCG CTG CCC AGC TGG ATT CGT CGA CAA GAC CTG CAG CCG CCC GGT
  766 GAG CAA CTG CGC CAG TGG CCC GTG CCA GAA CGG GGG CAC CTG CCT
  811 CCA GCA CAC CCA GGT GAG CTT CGA GTG TCT GTG CAA GCC CCC GTT
  856 CAT GGG TCC CAC GTG CGC GAA GAA GCG CGG GGC TAG CCC CGT GCA
  901 GGT CAC CCA CCT GCC CAG CGG CTA TGG GCT CAC CTA CCG CCT GAC
  946 CCC CGG GGT GCA CGA GCT GCC TGT TCA GCA GCC CGA GCA ACA CAT
  991 CCT GAA GGT GTC CAT GAA AGA GCT CAA CAA GAG TAC CCC TCT CCT
 1036 CAC CGA GGG ACA GGC CAT CTG CTT CAC CAT CCT GGG CGT GCT CAC
 1081 CAG CCT GGT GGT GCT GGG CAC CGT GGC CAT CGT CTT TCT CAA CAA
 1126 GTG CGA AAC CTG GGT GTC CAA CCT GCG CTA CAA CCA CAC GTT TCG
 1171 CAA GAA GAA GAA CCT CCT GTT GCA GTA TAA CAG CGG CGA GGA GCT
 1216 GGC GGT CAA TAT CAT CTT CCC CGA GAA GAT TGA CAT GAC CAC CTT
 1261 CAA CAA GGA GGC TGG TGA TGA GGA GAT CTA AGC AGC GTT CCC CAC
 1306 CCC CAC TCC CAG GCC CTT CAC CCC GAC CCC GAC CCA GGC CCT CTC
 1351 TAT TAC CGG GTT CCT TTA GAG CTC TCT ACC GAG TCT GGC TTT TTG
 1396 TGG TGG AGT TTG CTC TAT TGT GTG GAA TCG AGT GAA GCC TAT GCT
 1441 TAC ATA TAT TGT CTT GTG TTG CTG TGT GCC ATG CTA CCT CGC TAT
 1486 CTA AGA ACC CCT TCC TCC CTA TTA ATG CAT GAT AAT GAA TAA TAA
 1531 TAA TAA GAA TTT CAT CTC TAA ATG AAA AAA AAA AAA AAA AAA G
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Total number of bases is: 1573.

Consensus

dlk	C		PC NGG C D	Y	C C	PGF	G	C
Delta	C		PC NGGTC	Y	C C	GF	G	C
Serrate	C		PC NGGTC D	F	C C	G	G	C
TAN-1	C		PC NGGTC D	Y	C C	GF	G	C
Notch	C	S	PC NGGTC D	Y	C C	GF	G	C
Xotch	C		PC NGGTC D	Y	C C	GF	G	C
Lin-12	CL		C N G CI	Y	C C	GY	G	C

EGF mouse CPSSYDGYCLNGGVCMHIESDLSYT CNCVIGYSGDRC

FIGURE 4